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(54) Title: METHODS FOR PRODUCTION OF RECOMBINANT PLASMIDS			
(57) Abstract <p>The invention relates to a culture system for stable and high-level production of DNA contained on recombinant plasmids, and to the bacteria and plasmids which comprise the culture system. The bacterial cell chromosome is irreversibly altered, in one embodiment, so as to produce a substance toxic to the bacterium; in a second embodiment, so as to render the cells unable to synthesize and assimilate an essential metabolite, and, in a third embodiment, to be incapable of producing a required intracellular protein that does not lead to a secreted product. In every case, the recombinant plasmid includes genetic material which functionally complements the chromosomal alteration. If the DNA on the plasmid is to be used in therapeutic applications or for administration to eukaryotes, the genetic material will have no functional or structural equivalent in eukaryotic cells, and will not result in production of mRNA or a polypeptide that acts on any eukaryotic cell component. Any peptide produced is, desirably, not toxic to the bacterial cells.</p>			

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METHODS FOR PRODUCTION OF RECOMBINANT PLASMIDS

Background of the Invention

5 The combination of genetically engineered bacterial cells and recombinant plasmids are the foundation of industrial biotechnology. For example, they are used for production of industrially and medically important proteins such as enzymes, cytokines, growth hormones, and antigens or as live bacterial vaccines. The advent of DNA immunization and gene therapy technologies has added another dimension to the use of genetically engineered bacterial cells and their companion recombinant plasmids.

10 For the purpose of these technologies, expression of proteins in genetically engineered bacterial cells is no longer the principal objective, which is, instead, the replication and high-level production of structurally and genetically stable recombinant plasmids carrying foreign DNA. This is because the DNA, rather than a protein encoded therein, is the desired product for use in DNA immunization or gene therapy.

15 Consequently, the present invention relates to the use of genetically-engineered bacterial cells and their companion recombinant plasmids for cloning of foreign DNA. More particularly, the invention provides a method whereby foreign DNA suitable for use in DNA immunization and gene therapy can be replicated and produced in large quantities in these companion recombinant plasmids.

20 Early on during the development of recombinant DNA technology, it was realized that a major challenge to that emerging technology was the stable maintenance of recombinant plasmids in bacterial cells. It was also realized that this problem stems largely from the heavy metabolic burden imposed on genetically-engineered bacterial cells as a result of high-level expression of proteins which are of no value to them.

25 Consequently, when propagating genetically-engineered bacterial cells that have no incentive to maintain the recombinant plasmid, over time, plasmid-free bacterial cells appear at increasing frequency. Because of the heavier metabolic burden on plasmid-harboring bacterial cells, plasmid-free bacterial cells have higher growth rates. Accordingly, within a relatively short period of time, the bacterial culture can

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become dominated by plasmid-free bacterial cells, thus leading to a decreasing plasmid yield.

Since plasmid-harbouring bacterial cells are almost always at a growth disadvantage as compared to plasmid-free bacterial cells, any plasmid-free bacterial cells which arise during extended culture periods will eventually take over the fermentation bioreactor. In this regard, it is estimated that a 10% growth advantage for plasmid-free bacterial cells will result in bioreactor take-over within 150 hours of culture at a dilution rate of 1 hr/L even if the plasmid loss frequency is only 1×10^{-7} . These calculations underscore the need for a plasmid stabilization system that is 100% effective in preventing plasmid loss since it is not uncommon that bacterial cells are grown for 300 hours of continuous culture within industrial bioreactors.

These observations indicate that absence of selective pressure for maintaining recombinant plasmids in propagated genetically engineered bacterial cells results in a decreasing frequency of plasmid-harbouring bacterial cells, and that plasmid loss is further accentuated by the slower growth rate of plasmid-harbouring bacterial cells.

Several methods have been devised to enhance recombinant plasmid stability in bacterial cell populations. All these methods have a common underlying principle: the application of selective pressure to ensure the growth and multiplication of only those bacterial cells that are harbouring the recombinant plasmid.

In one of these methods, selective pressure is applied to bacterial cells by cloning the desired gene on a recombinant plasmid that also carries one or more genes specifying resistance to specific antibiotics. Thus, addition of the specific antibiotic(s) to a culture of growing bacterial cells ensures that only the ones harbouring the recombinant plasmid survive.

Although antibiotic resistance genes have been very useful and effective in providing a means of recombinant plasmid stability, their use has serious drawbacks. Firstly, addition of antibiotics in the culture medium during fermentation on an industrial scale is expensive. Secondly, in those cases where the mechanism of antibiotic resistance is based on secretion of an antibiotic-inactivating compound, some plasmid-free bacterial cells may survive because surrounding plasmid-harbouring

bacterial cells secrete enough of the antibiotic-inactivating compound into the culture medium so as to permit survival of both plasmid-harboring and plasmid-free bacterial cells. Thirdly, the use for DNA immunization and gene therapy of recombinant plasmid DNA containing antibiotic resistance genes is viewed unfavourably because
5 such genes might be incorporated into the animal genome, or into the genome of endogenous microflora. Fourthly, residual antibiotics that contaminate plasmid DNA (as a result of their addition to the culture medium) could provoke sensitivity and/or systemic allergic reactions in certain animals treated with such plasmid DNA.

As an alternative to the use of antibiotic resistance genes, several methods have
10 been devised to enhance recombinant plasmid stability and prevent accumulation of plasmid-free bacterial cells. The common thread to these methods is to make the survival of genetically-engineered bacterial cells dependent on a functional complementation system using a plasmid-borne gene.

These known methods can be divided into three groups depending on the type
15 of polypeptide encoded by the gene used. However, each of the known methods is either impractical in reducing the rate at which plasmid-free bacterial cells arise under industrial conditions and/or unsuitable for production of recombinant plasmids for use in eukaryotes, such as for DNA immunization and gene therapy. Each group of methods will be described in turn.

20 The first group comprises methods in which a chromosomal defect results in the failure to produce an essential nutrient and the plasmid-borne gene encodes an enzyme essential for the biosynthesis of this nutrient (e.g. an amino acid) that normally exists in commonly used bacterial growth medium (Dwivedi, C.P. *et al. Biotechnology and Bioengineering* (1982) 24:1465-1668; Imanaka, T. *et al. J Gen Microbiol* (1980)
25 118:253-261). These methods as set forth in the prior art require that the plasmid-harboring bacterial cells be grown on special and expensive synthetic medium lacking the amino acid in question. This is impractical in industrial bioreactor conditions.

The second group comprises methods wherein a chromosomal defect resides in defective production of an end-product that is required, and the plasmid-borne gene
30 encodes an enzyme that synthesizes this end-product, but where the end-product does

not exist in commonly used bacterial growth medium (Diderichsen, B. Bacillus Molecular Genetics and Biotechnology Applications (1986) pp.35-46; Ferrari, E. *et al. BioTechnology* (1985) 3:1003-1007; Galan, J.E. *et al. Gene* (1990) 94:29; Nakayama, K. *et al. BioTechnology* (1988) 6:696; Curtiss, R. *et al. Res Microbiol* (1990) 5 141:797).

To date, this method has focused on synthesis of amino acids that are incorporated into the bacterial cell wall. The utility of this approach in DNA immunization and gene therapy is hampered for the following reasons.

The enzymes that have been used in these situations to date (e.g., aspartate semialdehyde dehydrogenase (*asd*) or alanine racemase (*alr*)) catalyse the formation of 10 a small diffusible growth factor (aspartate semialdehyde and D-alanine, respectively), and the factor may accumulate in the culture medium under industrial bioreactor conditions. Such accumulation contributes to plasmid loss because of a cross-feeding effect in which plasmid-harboring bacterial cells (producing the small diffusible 15 growth factor) support the growth of plasmid-free bacterial cells. Therefore, to avoid such cross-feeding effects, this type of gene has been used with low-copy-number plasmids, i.e., of a type which occur as only 1 or 2 copies per bacterial cell. Clearly using low-copy-number recombinant plasmids is impractical for production of industrial quantities of plasmid DNA, where plasmids of a type resulting in high copy 20 numbers are desired. High-copy-number recombinant plasmids are those of a type which occur somewhere on the order of about 50 to several hundreds of plasmid copies per bacterial cell.

The *asd* gene as the plasmid-borne gene has another drawback. This drawback is related to the recent findings (Park, J.T. *Molecular Microbiology* (1995) 17:421- 25 426) that bacterial cells (e.g. *E. coli*) actually degrade approximately 50% of their peptidoglycan layer. The degradation product is a tripeptide consisting of L-alanine/D-glutamate/mesodiaminopimelic acid, which is reused by the bacterial cells to form peptidoglycan thus conserving energy that the bacterial cell would have expended synthesizing new tripeptide components of peptidoglycan. A high 30 proportion of this tripeptide is released into the culture medium and is available to be

taken up by neighbouring bacterial cells for incorporation into their own peptidoglycan layer. The *asd* gene encodes the first enzyme in the biosynthesis of the amino acid mesodiaminopimelic acid (dap) which is already included in this tripeptide. However, since bacterial cells can recycle their own peptidoglycan, and can secrete the tripeptide
5 containing the replaced amino acid dap, there is no selective pressure on the plasmid-harboring bacterial cells to maintain their plasmids.

The third group comprises methods wherein the plasmid-borne gene encodes a protein that has functional and structural counterparts in eukaryotic cells and/or is capable of acting upon a eukaryotic cell component. The use of such genes represents
10 a major safety concern because of the potential for the proteins encoded by these genes to function in eukaryotic cells and because of the potential for integration of the gene itself into the eukaryotic genome by homologous recombination. Examples include genes encoding proteins involved in the vital function of DNA replication (single strand DNA binding protein; Porter, R.D. *et al. BioTechnology* (1990) 8:47) or a
15 tRNA-related function (valine tRNA synthetase; Nilsson, J. and Skogman, G. *BioTechnology* (1986) 4:901-903).

The use as a marker of the gene encoding alanine racemase (*alr*) is also impractical for producing recombinant plasmid DNA for DNA immunization and gene therapy because this enzyme can function in eukaryotic cells. Alanine racemase
20 catalyses the conversion of L-alanine into D-alanine. Since eukaryotic cells contain L-alanine as a natural component of their biochemical make-up, the use of alanine racemase can interfere with the biochemical reactions involved in L-alanine biosynthesis in eukaryotic cells and could lead to the formation of an amino acid (D-alanine) that does not naturally exist in eukaryotic cells.

25 Recently, a meeting of the World Health Organization (WHO) devoted to issues of DNA immunization and gene therapy was convened (Nucleic Acid Vaccines, WHO, Geneva, as reported in Cichutek, K. *Vaccine* (1994) 12:1520; Robertson, J.S. *Vaccine* (1994) 12:1526; Smith, H. *Vaccine* (1994) 12:1515). At this meeting of experts in the field of DNA immunization and gene therapy, as well as experts from
30 regulatory authorities, a number of matters were declared crucial issues that have to be

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addressed in order to pave the way for these technologies to produce clinically useful products. These included structural and genetic stability of recombinant plasmids, the potential integration of recombinant plasmid DNA within host chromosomes, as well as the use of marker genes (e.g. antibiotic resistance genes) for selection and propagation of the desired plasmid-harboursing bacterial cells .

Thus, for the purpose of introducing foreign DNA into eukaryotes, such as for DNA immunization and gene therapy, there is a need for a system whereby genetically-engineered bacterial cells can be used for production of plasmid-borne foreign genes without the use of genetic material which itself can integrate within the eukaryotic genome or by virtue of its encoded product can function within eukaryotic cells or act upon any eukaryotic cell component.

In addition, improved methods for stable and high-productivity cloning in bacteria would be helpful simply for production of large quantities of desired DNA.

Disclosure of the Invention

The invention provides recombinant systems for production of large quantities of desired DNA, and in particular DNA that can be safely introduced into eukaryotes. The systems of the present invention offer advantages of efficiency and safety over art-known methods.

Thus, in one aspect, the invention is directed to culture systems and the components thereof which are designed for stable, high-level production of recombinant plasmids. In these culture systems, bacterial cells are employed wherein the bacterial cell chromosome is irreversibly modified to effect production of a substance toxic to the bacterial cells. The bacterial cells are modified to contain recombinant plasmids wherein the recombinant plasmids include genetic material that effects production of a second substance which neutralizes the toxicity of the first substance that would otherwise be toxic to the cells under the cell culture system conditions. The invention is directed also to the bacterial cells and plasmids useful in this cell culture system, and to methods to produce high levels of desired DNA using these materials. The plasmids employed in this system may further include a foreign

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DNA operatively linked to control sequences functional only in eukaryotes, whereby the foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

5 In a second aspect, the invention relates to cell culture systems for the stable, high-level production of recombinant plasmids wherein the bacterial cell chromosome of the bacteria in these cultures is irreversibly modified so as to render the cell incapable of producing an essential metabolite and also incapable of the uptake of the metabolite from the culture medium. The recombinant plasmid used in this system is a plasmid which includes genetic material which restores either the ability to synthesize the metabolite or the ability to take up the metabolite from the medium or both. This aspect of the invention also includes the bacterial cells and plasmids which are components of the cell culture system and methods to produce large quantities of DNA using the system. The plasmids may also be modified to contain a foreign DNA operably linked to control sequences functional only in eukaryotes, so that the foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

15 In still another aspect, the invention provides a culture system for stable and high-level production of recombinant plasmids for DNA immunization and gene therapy, comprising genetically engineered bacterial cells and recombinant plasmids. In this system, the bacterial cell chromosome is irreversibly altered and the bacterial cells are propagated under conditions such that the viability of the bacterial cells is dependant on the recombinant plasmid. The recombinant plasmid includes genetic material which functionally complements the chromosomal alteration, but the genetic material has no functional or structural equivalent in eukaryotic cells and does not produce any protein capable of acting upon any eukaryotic cell component. The protein encoded by the compensating genetic material, or any product thereof, is also incapable of being secreted by, or produced at levels toxic for, the bacterial cells. The recombinant plasmid is also adapted to include foreign DNA operatively linked to control sequences functional only in eukaryotes so as to effect expression of the foreign DNA only in eukaryotic cells, but not in prokaryotes. The invention is also directed to the bacterial cells and high-copy-number plasmids used in this cell culture system, as well as to methods to stably prepare foreign DNA for administration to eukaryotes using this system.

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In still other aspects, the invention is directed to methods to provide a desired foreign DNA to eukaryotic cells or a eukaryotic subject which method comprises contacting said cells or administering to said subject DNA prepared by the methods of the invention, or administering to the subject the bacterial cells containing the desired DNA.

Brief Description of the Drawings

Figure 1 shows the construction of recombinant plasmid pCB237.

Figure 2 shows the construction of a genetically engineered bacterial strain (CB101 *E. coli*) having a deletion in the chromosomal *galE* and *galT* genes.

Figure 3 is a schematic showing the cloning of the *murF* gene.

Figure 4 shows the construction of the recombinant plasmid pCB243.

Figure 5 shows the construction of a genetically engineered bacterial strain (CB1031 *E. coli*) having a deletion in the chromosomal *murF* gene.

Figure 6 is a diagrammatic representation of the construction of plasmids containing the *murF* gene.

Figure 7 shows plasmid yields for pMO106 produced in a *murF*-deficient strain at 42°C.

Figure 8 shows the stability of pMO106 in TKL-50 cells.

Figure 9 shows the *in vitro* transfection efficiency of a vector containing *murF*.

Figure 10 is a graphic representation of ELISA titers obtained from vectors containing antigens with and without the *murF* gene.

Figure 11 shows the nucleotide sequence of the temperature-sensitive *murF* gene derived from TKL-46.

Modes of Carrying Out the Invention

The present invention provides culture systems which permit the stable production of a desired DNA. In some instances, the desired DNA will be a foreign DNA whose expression in a eukaryotic cell is desired either for immunization or genetic therapy. This "expression" includes simple transcription as well as production of polypeptides. Thus, the foreign DNA for use in eukaryotes may be employed in antisense therapy, as well as in genetic therapy involving production of therapeutic proteins or markers.

When the foreign DNA is to be administered to a eukaryote, the amplified replicated DNA may be recovered and administered in the form of a pharmaceutical composition using standard formulation techniques. Suitable formulations for administering DNA include various excipients, for example, liposomes, dendrimers, aquasomes, cochleates, isotonic saline or PBS. The DNA can also be recovered and ligated into a retroviral vector. In addition to the DNA *per se*, the bacterial cells themselves containing the replicated DNA can be administered to a eukaryotic subject. The cells are chosen or manipulated so as to have the appropriate features which will permit the included DNA to locate in the nucleus of, for example, macrophage, so that expression of the foreign DNA may be effected. Thus, the prokaryotes administered must be able to exit the lysosome, disintegrate, and permit the DNA to enter the nucleus. Some bacterial hosts, such as *Shigella* and *Listeria* strains are innately capable of effecting these results. In addition to expressible DNA, vaccines which involve simply "naked DNA" have also been used successfully.

In general, administration of the DNA, either *per se* in a pharmaceutical composition or contained in the bacterial cells, is by injection; typically, intravenous, intramuscular, intradermal or subcutaneous. Administration may also be intranasal or oral, or by a particle bombardment technique. However, any effective systemic means of administration may be employed.

All of the culture systems of the invention rely on complementation of a destructive feature found in the bacterial chromosome by the effect of genetic material contained on a plasmid which is itself to be stably replicated in the bacterial culture. In

one embodiment, the bacterial chromosome produces a substance toxic to the bacterial cells and this substance is counteracted by a substance produced by genetic material on the plasmid. In a second embodiment, the bacterial chromosome is modified so as to render the cell incapable of producing an essential metabolite and also incapable of the uptake of this metabolite from the culture medium, and the recombinant plasmid restores one or both of these abilities. In either of these cases, an additional desired DNA sequence to be amplified may be included in the plasmid; in addition, this desired DNA may be operably linked to control sequences for expression exclusively in eukaryotic cells.

In a third approach, in this case for production of a foreign DNA operably linked to control sequences which effect expression exclusively in eukaryotes, particularly, the invention employs genetically engineered bacterial cells, the native chromosomal genome of which has been irreversibly altered. The alteration may consist of a modification of one or more chromosomal genes which alone or in combination are essential for cell viability in the conditions under which the bacterial cells are propagated or may consist of the insertion of one or more foreign genes that are detrimental to bacterial cell viability under such conditions.

The bacterial cells are then further modified by inclusion of a recombinant plasmid, preferably a high-copy-number recombinant plasmid (a type which occurs in a relatively high number of copies, from 50 to several hundred, per plasmid-harbouring bacterial cell). The recombinant plasmid is constructed to include genetic material which complements the above-mentioned chromosomal alteration. The introduction of the recombinant plasmid into the bacterial cells restores viability of the bacterial cells and ensures that only bacterial cells harbouring the recombinant plasmid can survive.

If the plasmid is to be employed for the production of an expression system operable only in eukaryotes for a foreign, desired DNA, the complementary genetic material must relate to one or more genes that have no functional or structural equivalent in the eukaryotic cells which are to be treated with the plasmid DNA. The complementary genetic material must not encode a polypeptide (or produce an mRNA) capable of acting upon any cell component of the eukaryotic cells which are to be

treated with the plasmid DNA. Furthermore, any factors or materials produced as a result of the presence of the complementary genetic material must be incapable of being secreted by, or of being produced at levels which are toxic for, the genetically engineered bacterial cells.

5 Thus, the complementary genetic material serves to ensure structural and genetic stability of the genetically engineered bacterial cells, by exerting selective pressure for survival only of plasmid-harboring bacterial cells. As stated above, the plasmid is preferably, but not necessarily, a high-copy number plasmid.

10 By cloning one or more foreign genes on the same recombinant plasmid, the genetically engineered bacterial cells can be used to produce large quantities of the plasmid DNA containing the foreign gene(s) for use in DNA immunization and gene therapy, including antisense therapy. The foreign genes preferably are incapable of being expressed in the genetically engineered bacterial cells, so as to avoid unnecessary metabolic burden or cell toxicity. This can be accomplished by operatively linking each
15 foreign gene to a promoter functional only in eukaryotes.

 Depending upon the foreign gene(s) cloned on the recombinant plasmid, the plasmid DNA can be used in DNA immunization and/or gene therapy. The plasmid DNA can be used for *in vivo* treatment, to convey one or more desired foreign genes to a eukaryotic host. For example, the foreign gene may be a mammalian gene
20 encoding a polypeptide required for the health or survival of a treated mammal. Or, the foreign gene may be a viral gene encoding a polypeptide against which it is desired to induce immunity in a treated animal. Alternatively, the expression system contained in the recombinant plasmid may produce an antisense mRNA for therapeutic treatment. Other examples will be apparent to those skilled in the art.

25 The invention will now be further described with reference to various preferred embodiments. However, the invention is not restricted to these embodiments, and one skilled in the art will readily appreciate alternative embodiments within the scope of the description of the features of the invention.

 One embodiment depends upon an amino-acid adding enzyme required for the
30 production of the bacterial cell wall.

Peptidoglycans are cell wall structures unique to bacterial cells. Accordingly, some of the genes encoding enzymes responsible for the biosynthesis or assembly of the peptidoglycan layer are excellent candidates for use as marker genes in recombinant plasmids intended for use in DNA immunization and gene therapy.

5 The peptidoglycan layer consists of several adjacent chains. Each chain consists of alternating units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues. Certain NAM residues on each chain are attached to a tetrapeptide whose composition varies slightly depending on whether the bacteria is Gram positive or Gram negative. The adjacent peptidoglycan chains are linked together by a peptide
10 bond that links the third amino acid, diaminopimelic (dap), of one tetrapeptide to the fourth amino acid, D-alanine, of the tetrapeptide attached to the NAM residue on the adjacent chain. The amino acid D-alanine is a unique component of the peptidoglycan of all bacteria. This amino acid is synthesized from L-alanine by the action of the enzyme designated alanine racemase (*alr*). The formation of this latter peptide linkage
15 is crucial to bacterial cell viability since in its absence the bacterial cells lyse in commonly used bacterial growth media.

Tetrapeptide formation requires the action of two different sets of genes, namely those that encode enzymes required for the biosynthesis of the individual amino acids of the tetrapeptides (e.g. enzymes required for biosynthesis of L-alanine,
20 D-glutamic acid, dap and D-alanine) and those that are required for the sequential addition and ligation of these amino acids to one another to form the tetrapeptide. The latter enzymes are called amino acid-adding enzymes. In general, the tetrapeptide sequence in the N→C direction is L-alanine/D-glutamic acid/diaminopimelic acid (dap)/D-alanine. The series of amino acid-adding enzymes comprises the L-Ala adding
25 enzyme that adds the amino acid L-alanine to the polysaccharide chain; the enzyme that adds the amino acid D-glutamic acid to the polysaccharide-linked L-alanine (*murD*) the enzyme that adds the amino acid dap to the L-alanine/D-glutamic acid dipeptide (*murE*), and the enzyme that adds D-alanine to the L-alanine/D-glutamic acid/dap tripeptide (*murF*).

Consequently, in one embodiment of the present invention, a system for production of plasmid DNA suitable for use in DNA immunization and gene therapy can be constructed by genetically engineering bacterial cells so as to render nonfunctional a chromosomal gene encoding one of the amino acid-adding enzymes (e.g. the *murF* gene). The viability of the genetically engineered bacterial cells is ensured by inclusion of a recombinant plasmid on which is cloned a functional *murF* gene.

Temperature-sensitive bacterial cells are known that contain a mutation in the gene encoding one of the amino acid-adding enzymes (e.g. *murF*). These cells cannot grow at nonpermissive temperatures, although they can synthesize the individual amino acid components of the tetrapeptide. The inability of these mutants to grow at nonpermissive temperatures is due to the fact that they cannot assemble a complete and functional tetrapeptide at this temperature. Thus, additional suitable bacterial host cells for use in the cell cultures of the present invention would include such existing strains cultured at nonpermissive temperatures.

There are several advantages to the use of the *murF* gene in the resulting system comprising genetically engineered bacterial cells including complementary recombinant plasmids. First, the *murF* enzyme is an intracellular and nondiffusible protein, and does not generate a product that is either toxic or secreted. Thus reduction in the number of plasmid-harboring bacterial cells because of the cross-feeding effect described previously is avoided. Second, the *murF* gene is unique to bacterial cells and has no functional or structural counterpart in eukaryotic cells, as demonstrated specifically for human cells below. Third, the *murF* gene has no substrate in eukaryotic cells. Thus there seems to be no risk of activity of the *murF* enzyme even if the *murF* gene were expressed in eukaryotic cells treated with the plasmid DNA. These advantages result in efficient recombinant plasmid stabilization and production under industrial fermentation conditions. Furthermore, the *murF*-based system is safe for use in DNA immunization and gene therapy.

Any of the four amino acid-adding enzymes discussed above can be used as the basis for the complementation system useful in the present invention. Thus, one can

alter the bacterial genome to delete the gene encoding the L-alanine-adding enzyme and furnish the gene encoding this enzyme on the plasmid; or the chromosome can be modified to disable it from synthesizing *murD*, *murE* or *murF* and supply the ability to produce the corresponding enzyme on the plasmid.

- 5 Although any one of these amino acid-adding enzymes can be chosen, there is a hierarchy which leads to a preferential selection of *murF* > *murE* > *murD* > L-Ala-adding enzyme. This is an efficiency-based consideration grounded in the manner in which cell wall degradation occurs when bacteria divide. Degradation inevitably takes place starting at the C-terminus of the tetrapeptide and may be incomplete. Thus, the
10 daughter cells generally need the *murF* component more desperately than they need *murE*, which is in turn more desperately needed than *murD* or L-alanine-adding enzyme.

- A second embodiment depends on rendering nonfunctional a chromosomal gene encoding an enzyme responsible for the synthesis of an amino acid that is present
15 naturally in commonly used bacterial media used in an industrial setting. Generally, such a chromosomal gene alteration is not a practical method for enhancing recombinant plasmid stability nor for high-level production of plasmid DNA. However, when additional safety precautions are incorporated, as exemplified below, this approach can be used for stable plasmid DNA production on an industrial scale.

- 20 For example, a chromosomal gene (designated *lysA*) which encodes an enzyme essential for the synthesis of the amino acid lysine is rendered nonfunctional. A second chromosomal gene (designated *lysP*) encoding a permease protein that is responsible for uptake of that amino acid from the environment or growth medium is also rendered nonfunctional. Such genetically engineered bacterial cells can no longer survive on
25 their own. They can survive when transformed with a recombinant plasmid carrying a functional gene for or equivalent to *lysA* or *lysP*. Thus, a genetically engineered bacterial cell that is defective in lysine uptake (due to a nonfunctional chromosomal *lysP* gene) and lysine biosynthesis (due to a nonfunctional chromosomal *lysA* gene) transformed with a recombinant plasmid carrying a functional or equivalent of *lysA*

gene provides an efficient system for stable and high level production of plasmid DNA for DNA immunization and gene therapy.

The plasmid-borne complementation gene in this example is the *lysA* gene since it is known that *lysA* gene has no structural or functional counterpart in eukaryotic cells. Furthermore, the *lysA* enzyme encoded by the *lysA* gene has no substrate in eukaryotic cells and as such this enzyme cannot function in eukaryotic cells even if it is expressed therein. Since lysine is an essential amino acid for all living microorganisms, including both Gram positive and Gram negative bacteria, this system provides a practical and versatile system for stable and high level production of plasmid DNA in any bacterial cell.

In a third embodiment, selective pressure is maintained by using a postsegregational killing mechanism. The naturally occurring *E. coli* plasmids R1 and F contain genetic loci appropriate for this approach. For the R1 plasmid, this locus is *parB* (Gerdes, K. *et al. PNAS* (1986) 83:3116-3120; Rasmussen, P.B. *et al. Mol Gen Genetics* (1987) 209:122-128). In the case of the F plasmid, this locus is *Flm* (Loh, S.M. *et al., Gene* (1988) 66:259-268). These loci mediate efficient recombinant plasmid stabilization by means of a postsegregational killing mechanism.

Both the *parB* and *Flm* loci consist of two small genes referred to as the *hok* (host killing) and *sok* (suppressor of host killing) genes in the case of *parB*, and *flmA* (host killing) and *flmB* (suppressor of host killing) in the case of the F plasmid. Thus, the *hok* and *flmA* genes are analogous in structure and function, and this *hok* and *flmB* genes are analogous in structure and function.

The *hok* and *flmA* gene products are small hydrophilic proteins (52 amino acids) that are potent host killing factors. The expression of *hok* gene is regulated by a small (about 100 base pairs) RNA molecule transcribed from the *sok* gene and which acts as an antisense RNA complementary to the mRNA of the *hok* gene. Similarly, the expression of the *flmA* gene is regulated by an approximately 100-base pair RNA molecule transcribed from the *flmB* gene which acts as an antisense RNA complementary to the mRNA of the *flmA* gene. The *hok* and *flmA* mRNAs are highly stable, whereas the *sok* and *flmB* RNAs are rapidly degraded. It is the differential

stability of the two RNA species in each case which results in the mechanism of bacterial cell killing. When a bacterial cell containing a plasmid with the *parB* locus loses such a plasmid, the prolonged persistence of the *hok* mRNA leads to synthesis of *hok* protein, thus ensuring a rapid and selective killing of newly formed plasmid-free bacterial cells. Similarly, when a bacterial cell containing a plasmid with the *Flm* locus loses the plasmid, the prolonged persistence of the *flmA* mRNA leads to the synthesis of the *flmA* protein, thus ensuring rapid and selective killing of newly formed plasmid-free bacterial cells.

The combined effect of *hok* and *sok* genes or of the *flmA* and *flmB* genes can be used to advantage in constructing a system for use in plasmid DNA production for use in DNA immunization and gene therapy. The utility of these systems could be hampered by the potential for killing eukaryotic cells such as those of mammalian hosts injected with plasmid DNA containing the *hok/sok* or *flmA/flmB* combination during DNA immunization or gene therapy. Accordingly, only the *sok* gene (100 bp) or *flmB* gene (100 bp) is included in the recombinant plasmid, whereas the *hok* gene or *flmA* gene is integrated in the bacterial cell chromosome.

For the plasmid production system based on the *hok/sok* or *flmA/flmB* combination, a bacterial strain is genetically engineered in which the gene encoding the *hok* or *flmA* protein is inserted in a non-essential region of the bacterial chromosome (e.g. in the *lacZ* gene). Genetically engineered bacterial cells are then transformed with a recombinant plasmid in which the only marker is the corresponding *sok* or *flmB* gene. So long as the recombinant plasmid remains in the bacterial cells, the *sok* or *flmB* gene on the recombinant plasmid serves to regulate the expression of the *hok* or *flmA* gene and the bacterial cell thrives. As soon as the recombinant plasmid carrying the *sok* or *flmB* gene is lost, the bacterial cell dies because of the killing factor produced by the chromosomal *hok* or *flmA* gene.

This system offers the following specific advantages for production of plasmid DNA. First, the system is not limited in its use to a particular bacterial strain. Second, since only 100 bp of DNA encoding the *sok* or *flmB* gene will be used in the recombinant plasmid, a much smaller and more compact plasmid can be constructed.

This lends itself to higher plasmid yield and provides the ability to clone more than one foreign gene on the recombinant plasmid.

One skilled in the art will appreciate other combinations according to the invention of genetically engineered bacterial strains and recombinant plasmids (which
5 can serve as a vector for foreign DNA) wherein the recombinant plasmid includes DNA complementary to the altered (i.e. added or rendered nonfunctional by modification, deletion, or inactivation) chromosomal DNA and which accordingly serves as a marker for presence or absence of the recombinant plasmid, as well as selective pressure to maintain the recombinant plasmid in the genetically engineered
10 bacterial cell.

The present invention will be illustrated in detail in the following examples. These examples are included for illustrative purposes, and should not be considered to limit the present invention.

15

Example 1

Construction of Chromosomally Altered Host CB102

The peptidoglycan layer is absolutely required for bacterial cell viability under ordinary circumstances, as it protects the fragile cytoplasmic membrane from osmotic shock. However, under certain circumstances, bacterial cells that have a defect in
20 peptidoglycan synthesis can still survive in certain types of media such as those supplemented with sodium chloride or sucrose. The reason for the ability of these bacterial cells to survive in these types of media is attributed to the action of these supplementary compounds as osmotic stabilizers or due to their ability to induce colanic acid production which acts as an osmotic stabilizer. Thus, in a preferred
25 embodiment bacterial cells are genetically engineered so as to be absolutely dependent on the presence of an intact peptidoglycan layer regardless of the type of media in which the bacterial cells are grown. The ability of these bacterial cells to synthesize colanic acid is, in this embodiment, abolished in addition to introducing a mutation or a deletion that results in a defective peptidoglycan assembly.

Colanic acid is a polymer composed of glucose, galactose, fucose, and glucuronic acid. Since galactose is one of the colanic acid components, one way to abolish the ability of bacterial cells to produce colanic acid is to inhibit the ability of bacterial cells to synthesize and utilize galactose. In this example, this is achieved by introducing irreversible deletions in the *galE* and *galT* chromosomal genes involved in galactose utilization. The resulting bacterial strain is then subjected to a deletion in the *murF* chromosomal gene to produce a strain whose viability is dependent on complementation with a recombinant plasmid carrying a functional *murF* gene, as follows:

First, the JM105 *E. coli* strain containing a deletion in the *galE* and *galT* genes was constructed. To clone the *galE* and *galT* genes, two oligonucleotide primers (upstream and downstream) were synthesized based on the known nucleotide sequences of the *galE* and *galT* genes. To facilitate cloning, an *Xba*I site was engineered at the end of each of these primers. The upstream primer (designated gal-3) corresponded to the 5' end of the *galE* gene and had the following nucleotide sequence:

5'gctctagaggctaaattctgtgttaaacga3.

The downstream primer (designated gal-4) corresponded to the 3' end of the *galT* gene and had the following nucleotide sequence:

5'gctctagatctgccagcatttcataaccaa3'.

Primers gal-3 and gal-4 (100 pmoles each) were combined with 2 µl of an overnight culture of JM105 bacterial cells. To this mix was added 4 µl of a 5 mM deoxynucleoside (dNTP's) solution, 1 µl of 100 mM MgSO₄, 5 µl of 10X Vent reaction buffer and 1 µl of Vent DNA polymerase (purchased from NEB Biolab). The reaction mix was amplified for 30 cycles using a cycling profile as follows: Melting: 94°C for 1 minute, Annealing: 55°C for 1 minute, and Extension: 72°C for 2 minutes. Following amplification, the reaction mix was analyzed by electrophoresis through 1% agarose. A single band of the expected size (approximately 2 Kilobase pairs; corresponding to the entire *galE* and *galT* genes) was evident following ethidium bromide staining of the DNA present in the polymerase chain reaction (PCR) reaction

mix. The above DNA band was excised and purified away from the agarose using the GeneClean kit (obtained from BioCan). As illustrated in Figure 1, the purified DNA fragment was treated with T4 polynucleotide kinase (Pharmacia) and ligated into the HincII site of pTZ18 plasmid. pTZ18 plasmid containing the *galE* and *galT* genes was
5 designated pCB233.

An internal deletion within *galE* and *galT* genes was constructed as further illustrated in Figure 1. A pTZ18 plasmid was cut with HindIII enzyme, treated with Klenow enzyme, and self-ligated to produce plasmid pCB234 in which the HindIII site no longer existed. Plasmid pCB233 was cut with EcoRI and PstI enzymes to retrieve
10 a DNA fragment encompassing the entire *galE* and *galT* genes. This latter fragment was ligated into pCB234 that was also cut with EcoRI and PstI enzymes. The ligation event led to the isolation of a plasmid designated pCB235. Plasmid pCB235 was cut with HindIII and NcoI enzymes to delete an internal portion of the nucleotide sequence within the *galE* and *galT* sequences, treated with Klenow enzyme, and then self-ligated
15 to produce plasmid pCB236. Plasmid pCB236 was cut with EcoRI and PstI enzymes to retrieve the shortened *galE* and *galT* genes. The DNA fragment encompassing the shortened *galE* and *galT* genes was treated with T4 DNA polymerase enzyme. At the same time, the suicide vector plasmid designated pCVD442 (J.B. Kaper, University of Pennsylvania) was cut with XbaI enzyme and then treated with Klenow enzyme. The
20 DNA fragment encompassing the shortened *galE* and *galT* genes was then ligated into the above pCVD442 and transformed into *E. coli* SY327 to produce plasmid pCB237. Plasmid pCB237 was then transformed into *E. coli* SM10 and the latter bacterial cells containing pCB237 were selected for further use as described below.

The *galE* and *galT* deletions were introduced into JM105 *E. coli* as illustrated
25 in Figure 2. SM10 bacterial cells carrying plasmid pCB237 were used to transfer the irreversibly nonfunctional (i.e. with internal deletion) *galE* and *galT* genes into *E. coli* strain JM105 by the conjugation protocol described by Donnenberg, M. and Kaper, J *Infection and Immunity* (1990) 59:4310-4317. The incorporation of the irreversibly nonfunctional *galE* and *galT* genes into the chromosome of JM105 bacterial cells and
30 the replacement of full-length wild type *galE* and *galT* genes of the latter bacterial cells with the irreversibly nonfunctional *galE* and *galT* genes was verified by PCR analysis

- 20 -

of JM105 chromosomal DNA. The JM105 *E. coli* bacterial strain containing an internal deletion in *galE* and *galT* genes was designated CB101.

To produce a deletion in the *murF* locus, first the chromosomal *murF* gene from JM105 *E. coli* was isolated using the polymerase chain reaction (PCR). Two oligonucleotide primers were synthesized based on the known sequence of the *murF* gene. These primers were designated *murF1* and *murF2* and their nucleotide sequences were as follows:

Upstream primer (*murF1*):

5'cgagcactgcgagagatgattagcgtaacccttagccaactt3' and

Downstream primer (*murF2*):

5'cagcgcggtgcagcaggctgacagtggcgcgga3'.

The *murF* gene appears to be transcribed under the control of the *murE* promoter, consequently the above PCR primers were designed so as to allow inframe fusion of the *murF* coding sequence to the *murE* promoter. For this purpose, the *murE* gene including its promoter element was isolated from JM105 *E. coli* using PCR primers designated *murE1* and *murE2*. The nucleotide sequences of these primers were as follows:

Upstream primer (*murE1*):

5'gccggatccgcgccggtctttggtgccca3', and

Downstream primer (*murE2*):

5'aagggatccgctaatacatgcaatcacc3'.

As illustrated in Figure 3, following PCR amplification, the *murE* gene was cloned into plasmid pBR322 to produce a plasmid designated pCB238. The *murF* gene was amplified from JM105 bacterial cells using primers *murF1* and *murF2*.

Plasmid pCB238 was digested with *NruI* enzyme to remove the *murE* gene coding sequence, leaving behind the *murE* promoter sequences. The amplified *murF* gene was then ligated to the plasmid pCB238 that had been digested with *NruI* enzyme and the resulting plasmid was designated pCB239.

Plasmid pCB239 is digested with appropriate enzymes to excise a DNA fragment encompassing the *murE* promoter fused to the *murF* gene, and the fragment is then cloned into plasmid pACYC184 to produce plasmid pCB240.

In order to construct an *E. coli* strain with an irreversible alteration (i.e. deletion) in its *murF* gene, a plasmid in which a deleted *murF* gene is cloned along with DNA sequences derived from upstream and downstream flanking sequences is constructed. For this purpose, an oligonucleotide primer is designed based on the known sequences of the *murE* gene (upstream of *murF* gene) and a second oligonucleotide primer is designed on the basis of the known sequence of the *OrfY* gene (downstream of the *murF* gene). The upstream primer is designated *murE1* and had the following nucleotide sequence:

5'gccggatccgcccggcttttggtgcca3'.

The downstream primer is designated *OrfY-1* and has the following nucleotide sequence:

5'taacgccagcgaacctacatc3'.

Primers *murE1* and *OrfY-1* are used in the PCR reaction to amplify the *murF* gene with flanking sequences derived from the *murE* and the *OrfY* gene. As illustrated in Figure 4, the amplified DNA fragment is cloned in plasmid pBR322 to produce plasmid pCB241. Plasmid pCB241 is digested with appropriate enzymes to remove most of the *murF* gene leaving only a small portion of the *murF* gene sequence flanked by a sequence derived from the *murE* gene on one side and a sequence derived from the *OrfY* gene on the other side. Following digestion, the plasmid is self-ligated to produce plasmid pCB242. The DNA fragment encompassing the sequences derived from the *murE* part of *murF* and *OrfY* genes is retrieved from plasmid pCB242 by digestion of the latter with appropriate enzymes. The DNA fragment carrying the latter sequences is then ligated into the suicide vector pCVD442 and transformed into SY327 *E. coli* cells. The latter bacterial cells are used to prepare plasmid pCVD442 containing the flanking sequences and this latter plasmid is designated pCB243. Plasmid pCB243 is then transformed into *E. coli* SM10 cells to produce a population

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of bacterial cells suitable for use in transfer of the *murF* deletion into the *E. coli* strain CB101.

As illustrated in Figure 5, SM10 bacterial cells carrying plasmid pCB243 are used to transfer the irreversibly nonfunctional (i.e. with internal deletion) *murF* gene into *E. coli* strain CB101 transformed with plasmid pCB240 by the conjugation protocol described above (Donnenberg and Kaper 1990). The incorporation of the irreversibly altered *murF* gene into the chromosome of CB101 bacterial cells and the replacement of full-length wild type *murF* gene of the latter bacterial cells with the irreversibly altered *murF* gene is verified by PCR analysis of CB101 chromosomal DNA. The CB101 bacterial strain containing an internal deletion in the *murF* gene is designated CB102.

Bacterial strain CB102 can then be transformed with plasmid pCB239 to displace plasmid pCB240. Bacterial strain CB102 carrying plasmid pCB239 is designated CB103.

Example 2

Construction of an Alternative *murF* Deficient Bacterial Host, TK-48

Certain temperature-sensitive bacterial cell mutants containing a mutation in the *murF* gene, such as TKL-46, can grow at 30°C, but cannot grow at the nonpermissive temperature of 42°C. The inability of these mutants to grow at nonpermissive temperatures is due to the fact that they cannot assemble a complete and functional cell wall tetrapeptide due to the mutation in the *murF* gene. These mutants can be used as bacterial hosts in the cell culture systems of the invention.

A system for production of recombinant plasmids suitable for use in DNA immunization and gene therapy can thus be constructed by inclusion, in these temperature-sensitive bacterial cells (e.g. *E. coli* strain TKL-46 or its derivatives), of a recombinant plasmid containing a *murF* gene functional at 42°C. By growing the bacterial cells at 42°C, only cells carrying the recombinant plasmid can survive.

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In this example, a more preferred derivative of TKL-46, useful as a host, was prepared. *E. coli* strain TKL-46 (Lugtenberg *et al.*, *J Bacteriology* (1972) 110:35-40) was obtained from Coli Genetic Stock Center (CGSC). TKL-46 bacterial cells were first grown at 30°C in LB broth containing the antibiotic nalidixic acid to select for a
5 nalidixic acid-resistant strain, designated TKL-47. TKL-47 cells were rendered *recA*-negative by conjugation with the (nalidixic acid-sensitive, *recA*-negative) bacterial strain JC10240 in order to abolish the ability of the cells to perform homologous recombination. Thus their chromosomes will not acquire any genetic material from introduced plasmid DNA. *RecA*-negative cells derived from TKL-47 cells were
10 designated TKL-48.

TKL-48 bacterial cells were grown at 30°C and then used for preparation of competent bacterial cells. Competent TKL-48 bacterial cells are transformed with plasmid pCB239 carrying a functional *murF* gene to produce a bacterial strain designated TKL-49. TKL-49 bacterial cells are then grown at 42°C as a method for
15 recombinant plasmid maintenance and plasmid DNA production.

As an alternative to CH102 or TKL-48 bacterial cells as hosts, additional improved forms are constructed by modifying the *murF* gene in the chromosomes of cells that have native qualities that are desirable. For example, some *E. coli* strains are deficient in enzymes, such as endonucleases which degrade plasmid DNA. If the
20 chromosomes of these hosts can be altered to provide, for example, the temperature-sensitive form of *murF*, these hosts provide advantageous alternatives to TKL-48.

The temperature-sensitive *murF* gene from TKL-46 was obtained by amplifying the appropriate sequence using PCR with *murF3* and *murF4* as primers:

murF3 (Upstream primer):

25 5'gccggatcccgatcgcggtcacgggtggcgcg3'

murF4 (Downstream primer):

5'gaagatctcagcgcggtgcagcaggctgacagtggcgcg3'

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The amplified nucleotide sequence was cloned into the BamHI site of pUC19 and the nucleotide sequence was determined using the dideoxynucleotide method. The complete nucleotide sequence of the amplified gene is shown in Figure 11, and differs from the wild-type gene as positions 862 and 990 as shown in Figure 11. The replacement of G with A at position 862 results in threonine rather than alanine; the addition of the GAC codon at position 990 results in addition of an aspartic acid residue to the peptide sequence.

The host strain with desirable characteristics is then altered by replacing the wild-type *murF* gene with the temperature-sensitive form typically by homologous recombination to obtain the improved bacterial host.

Thus, either CB102 or TKL-48 or other desired bacteria modified to contain the temperature-sensitive *murF* gene, which have irreversible chromosomal mutations in the *murF* gene, rendering the gene or the encoded *murF* protein nonfunctional under the culture conditions employed, can be used as suitable hosts where the introduced plasmid has a *murF* gene functional under the culture conditions employed. The production levels can be compared to production of foreign DNA contained on plasmid pBR322 in which a marker gene encodes ampicillin.

Example 3

Construction of Additional *murF*-Containing Plasmids

Using the *murE1* and *murE2* primers described in Example 1, the chromosomal *murE* gene was PCR amplified from *E. coli* JM105 genomic DNA and cloned into the BamHI site of pUC18. The resulting plasmid, pMO101, was cut with *NruI* which removes most of the *murE* gene, except for the *murE* promoter and approximately 330 bp of the *murE* 3' end. The *murF* gene, amplified as described in Example 1, was ligated into the *NruI* site of pMO101, and the ligation mixture transformed into *E. coli* JM109. Two plasmids, designated pMO102 and pMO106 were identified, which contain the *murF* coding sequence in opposite orientations. pMO106 contains the *murF* gene in the correct orientation operably linked to the *murE* promoter and expresses the *murF* protein; pMO102 contains the wrong orientation for *murF*, and

thus does not express the gene. A summary of the construction of pMO102 and pMO106 is shown in Figure 6.

Additional plasmids containing the *murF* gene were constructed as follows:

pHW203 was constructed as a system for testing *in vitro* transfection efficiency into eukaryotic cells. pCMVB, available from Clontech, which contains the β -galactocidase gene under control of a promoter operable in mammalian cells was cut with *EcoRI*, treated with Klenow, and ligated with the *BamHI*, Klenow-filled fragment from pMO106 that contained the *murF* gene. The resulting plasmid was cut with *BstHI* to remove the ampicillin-resistance gene and religated. The religated product was transformed into TKL-48 and an isolated colony, containing the plasmid pHW203, the colony designated TKL-52, was used for large-scale production of the plasmid.

The plasmid pCB253 was constructed in order to evaluate whether the *murF* gene had any effect on DNA immunization in eukaryotic hosts. The host plasmid, pSLRSGIV.md1, obtained from R. Braun contains the gene encoding glycoprotein IV (GIV) from bovine herpes virus under control of the Rous Sarcoma Virus promoter; therefore, this plasmid contains an expression system for production of an immunogen in vertebrates. The *BamHI* DNA fragment from pMO106 containing the *murF* gene under control of the *murE* promoter was ligated into the *Msc* site of pSLRSGIV.md1 and the ligation mixture transformed into JM109 cells (obtained from New England Biolabs). The plasmid isolated from successful transformants was digested with *BstHI* to remove the ampicillin resistance gene, religated and then transformed into TKL-48 to obtain the product plasmid pCB253. The pCB253 colony, designated TKL-51, was used for large-scale preparation of this plasmid.

In the foregoing paragraphs, TKL-48 was transformed with pMO102, pMO106, pCB253 or pHW203 by mixing competent cells with plasmid DNA and incubating on ice for 30 min. The cells were then heat-shocked at 42°C for 90 sec and then incubated in 1 ml of LB broth at 30°C for 2 hrs. The cells were then plated on LB/0.1% thymine plates and incubated at 42°C overnight. The transformed cell lines obtained were designated as follows:

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pMO102 - TKL-49A;

pMO106 - TKL-50;

pCB253 - TKL-51;

pHW203 - TKL-52.

5 Of course, TKL-49A cells produce the *murF* gene product only 30°C, and they are used as a control to assess plasmid yield in the presence of ampicillin as the selectable marker at 30°C. Plasmid yield could be assessed at 42°C without addition of ampicillin for the remaining cell lines.

10 Example 4

Plasmid Yield

TKL-50, -51, and -52 cells were used to assess plasmid yield when cells are grown at 42°C without addition of ampicillin, TKL-49A cells were used as controls. Single colonies from LB/thymine plates were picked and inoculated into 2 ml of
15 TB/thymine broth and incubated for 6 hr at 42° (30°C in presence of ampicillin for TKL-49A). Cells were then inoculated into 10 ml TB/thymine broth, grown for four more hours, then inoculated into 250 ml of TB/thymine broth and grown for an additional 12 hr. Plasmids were extracted from cells using Quiagen columns as recommended by the manufacturer, and plasmid yield determined by calculating O.D.
20 260 and 280. Purity of plasmids was determined by analysis of plasmid aliquote on agarose gels.

Plasmid yield was also determined in a 10-liter fed-batch oxygen enriched fermentation in a 14-liter NBS Microferm apparatus, in TB broth supplemented with 0.01% thymine at agitation speed of 200 rpm, aeration flow of 0.6VVM (6 l/min,
25 5 psig) and pH maintained at 7.2. Plasmid yield was determined following extraction of plasmid DNA from aliquots of culture medium using the Quiagen column method.

Yields from cells grown in shake flasks (3 preparations) or a 10-liter fermentor (two runs) is shown in Table 1.

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Table 1				
Plasmid yields from cells grown in shake flasks and a 10-liter fermentor				
Strain/Plasmid Combination	Selection Pressure	Absolute Plasmid Yield (mg/liter) ^a		Specific Plasmid Yield (mg/gm dry weight) ^b
		shake flasks	fermentor	
TKL-49/pMO102	30°C/Ampicillin	2.67 ± 0.16	43.85	3.3
TKL-50/pMO106	42°C/ <i>murF</i>	6.13 ± 0.71	144	6.788
TKL-51/pCB253	42°C/ <i>murF</i>	8.61 ± 0.51	107	N/D ^d
TKL-52/pHW203	42°C/ <i>murF</i>	6.14 ± 2.2	N/D	N/D

^aPlasmid yield from shake flasks and fermentor runs are extrapolated from aliquots of culture volumes (5, 10, and 25 ml) used for preparation of plasmid DNA.

^bgram dry weight per liter of culture (g/L) was determined from the equation:

$$\text{g/L} = 0.08 + 0.63(\text{O.D.}660)$$

- 5 ^cYield is the average of two fermentations for pMO102 and pMO106. Yield of pCB253 is derived from one fermentation run.

^dNot Determined

Figure 7 shows the absolute and specific plasmid yield determined for plasmid pMO106 produced in TKL-50 strain.

10

Example 5

Plasmid Stability

- Stability of plasmid pMO106 in TKL-50 strain was assessed over 170 generations of growth under different selection pressures for plasmid maintenance as follows. Cells were grown in TB/0.1% thymine broth at either 30°C or 42°C.
- 15 Samples were taken after different numbers of generations at the relevant temperature, diluted and plated on TB/thymine plates and incubated at 30°C. Colonies were then replicated onto TB/thymine/ampicillin plates and incubated at 30°C or 42°C. The

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percent of plasmid-containing cells was determined by comparing the number of colonies growing in the presence and absence of ampicillin at 30°C or 42°C. Presence of plasmids was further verified by the small scale alkaline lysis method for plasmid preparation.

- 5 Plasmid pMO106 was gradually lost from cells grown at 30°C, but was retained in 100% of the cells grown at 42°C for 170 generations of growth as shown in Figure 8. The integrity of plasmid pMO106 was confirmed by restriction analysis.

TKL-49A cells were also tested by growing in the presence of ampicillin at 30°C; plasmid pMO102 was retained in 100% of the cells for 120 generations.

10

Example 6

Effectiveness of In Vitro Transfection

- Plasmid pCMVB and pHW203, described above were used in this assay. 2 µg of each plasmid were mixed separately with varying amounts of lipofectamine (Gibco/BRL) and transfected into the mouse fibroblast L-929 cells according to the manufacturer's recommendation. The efficiency of transfection was determined in an X-gal colorimetric assay by counting the number of cells expressing β-galactosidase relative to the total number of cells per well.
- 15

- Similar transfection efficiencies were found for both plasmids as shown in Figure 9.
- 20

Example 7

Effect of the *murF* Gene on Immunization

- Groups of ten mice were immunized twice (two weeks apart) intramuscularly with 100 µg each of either plasmid pCB253, pSLRSG1V.md1 (described above) or with a null vector. At one and three weeks following the second injection, the mice were bled and their sera analyzed for the presence of antibodies specifically
- 25

immunoreactive for the GIV glycoprotein using an ELISA assay. Similar antibody titers were found in both cases, as shown in Figure 10.

Example 8

5 Lack of *murF* Homology to Human Sequences

Searches of nucleotide and amino acid sequence data bases showed that sequences associated with the *murF* gene/protein have no homology to any known human gene/protein sequences. To confirm this lack of homology, human genomic DNA, purchased from Promega Corporation was digested with *EcoRI* and *BamHI* and
10 spiked with pMO106 cut with *BamHI*. The genomic DNA was electrophoresed in 0.7% agarose gels along with the *BamHI* fragment containing the *murF* gene from pMO106, the *murF* gene coding sequence *per se*, and an *EcoRI* linearized plasmid pMO106. After transfer to nylon membranes using capillary transfer, the DNAs were fixed by ultraviolet light cross-linking according to the manufacturer's instructions.
15 The membranes were prehybridized for 8 hours in 6xSSC, 0.5% sodium dodecosulfate at 50°C and then hybridized for 12 hours with ³²PdCTP labeled full-length *murF* DNA probe. The blots were washed twice in 2xSSC, 0.5% SDS for 30 minutes at 22°C, followed by two washings for 30 minutes in 1xSSC, 0.5% SDS at 50°C. After drying and exposure to X-ray films for 16 hours-2 weeks, no hybridization signal with human
20 genomic DNA was detected.

Claims

1. A culture system for stable, high-level production of recombinant plasmids comprising genetically engineered bacterial cells and number recombinant
5 plasmids,

wherein the bacterial cell chromosome is irreversibly modified to effect production of a first substance toxic to bacterial cells, and

wherein the recombinant plasmid includes genetic material which effects production of a second substance effective to neutralize the toxicity of said first
10 substance under the culture conditions of said culture system, and optionally further includes foreign DNA operatively linked to control sequences functional only in eukaryotes whereby said foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

15 2. The cell culture system of claim 1 wherein the first substance is the product of the *hok* gene and the second substance is the product of the *sok* gene or wherein

the first substance is the product of the *flmA* gene and the second substance is the product of the *flmB* gene.

20

3. A stable recombinant plasmid for use with a bacterial cell said cell having its chromosome irreversibly modified to effect production of a first substance toxic to bacterial cells,

wherein the recombinant plasmid includes genetic material that effects
25 production of a second substance which neutralizes the toxic effects of said first substance; and optionally further includes foreign DNA operatively linked to control sequences functional only in eukaryotes whereby said foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

4. The plasmid of claim 3 wherein the first substance is the product of the *hok* gene and the second substance is the product of the *sok* gene or wherein

the first substance is the product of the *flmA* gene and the second substance is
5 the product of the *flmB* gene.

5. A bacterial cell having its chromosome irreversibly modified to effect production of a first substance toxic to bacterial cells which cell further includes the plasmid of claim 3 or 4.

10

6. A method to replicate DNA contained in a plasmid which method comprises culturing the cells of claim 5 under conditions wherein the viability of said cells is dependent on the presence of said plasmid in said cells.

15

7. A culture system for stable, high-level production of recombinant plasmids comprising genetically engineered bacterial cells and recombinant plasmids,

wherein the bacterial cell chromosome is irreversibly modified so as to render the cell incapable of producing an essential metabolite and also incapable of the uptake of said metabolite from a culture medium, and

20

wherein the recombinant plasmid includes genetic material which restores either the ability to synthesize said metabolite or the ability to take up the metabolite from the medium or both and which optionally further includes foreign DNA operatively linked to control sequences functional only in eukaryotes whereby said foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

25

8. The culture system of claim 7 wherein the essential metabolite is lysine.

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9. The culture system of claim 8 wherein the inability to synthesize lysine is effected by a mutation in the *lysA* gene and/or the inability to take up lysine from the medium is effected by a disruption in the *lysP* gene.

5 10. A stable recombinant plasmid for use with a bacterial cell having its chromosome irreversibly modified so as to render the cell incapable of producing an essential metabolite and also incapable of the uptake of said metabolite from a culture medium,

10 wherein the recombinant plasmid includes genetic material which restores either the ability to synthesize said metabolite or the ability to take up the metabolite from the medium or both and which optionally further includes foreign DNA operatively linked to control sequences functional only in eukaryotes whereby said foreign DNA is expressed in eukaryotic cells, but not in prokaryotic cells.

15 11. The plasmid of claim 10 wherein the essential metabolite is lysine.

12. The plasmid of claim 11 wherein the inability to synthesize lysine is effected by a mutation in the *lysA* gene and/or the inability to take up lysine from the medium is effected by a disruption in the *lysP* gene.

20

13. A bacterial cell having its chromosome irreversibly modified so as to render the cell incapable of producing an essential metabolite and also incapable of the uptake of said metabolite from a culture medium which includes the plasmid of any of claims 10-12.

25

14. A method to replicate DNA contained in a plasmid which method comprises culturing the cells of claim 13 under conditions wherein the viability of said cells is dependent on the presence of said plasmid in said cells.

15. A culture system for stable high-level production of recombinant plasmids comprising genetically engineered bacterial cells and recombinant plasmids, wherein the bacterial cell chromosome is irreversibly altered and the bacterial cells are propagated under conditions such that the viability of the bacterial cells is dependent on the recombinant plasmid, and wherein the plasmid includes genetic material which functionally complements the chromosomal alteration,

wherein said genetic material has no functional or structural equivalent in eukaryotic cells; and

wherein any protein expressed by said genetic material is incapable of acting upon any eukaryotic cell component; and

wherein any protein expressed by said genetic material or product resulting from the presence of said protein is not secreted by or toxic to said bacterial cells; and

wherein the plasmid further includes foreign DNA operatively linked to control sequences functional only in eukaryotes, whereby the foreign DNA is expressed in eukaryotic cells, but not in bacterial cells.

16. The cell culture system of claim 15 wherein the chromosome is altered so as to disrupt the synthesis of the bacterial cell wall.

17. The cell culture system of claim 16 wherein the chromosome is altered so as to result in the inability of said bacterial cells to synthesize an enzyme that catalyzes a step in the biosynthesis of said cell wall.

18. The cell culture system of claim 17 wherein said enzyme catalyzes a reaction selected from the group consisting of the addition of L-alanine to a cell wall polysaccharide (L-Ala-adding enzyme); the addition of D-glutamic acid to the L-alanine attached to the polysaccharide chain (*murD*); the addition of dap to the

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L-alanine/D-glutamic acid dipeptide (*murE*); and the addition of D-alanine to the L-alanine/D-glutamic acid/dap tripeptide of the bacterial cell wall (*murF*).

19. A stable recombinant plasmid for use with a bacterial cell having its
5 chromosome irreversibly altered so that when the bacterial cells are propagated under suitable conditions, the viability of the bacterial cells is dependent on said plasmid, and wherein the plasmid includes genetic material which functionally complements the chromosomal alteration,

10 wherein said genetic material has no functional or structural equivalent in eukaryotic cells; and

wherein any protein expressed by said genetic material is incapable of acting upon any eukaryotic cell component; and

wherein any protein expressed by said genetic material or product resulting from the presence of said protein is not secreted by or toxic to said bacterial cells; and

15 wherein the plasmid further includes foreign DNA operatively linked to control sequences functional only in eukaryotes, whereby the foreign DNA is expressed in eukaryotic cells, but not in bacterial cells.

20 20. The plasmid of claim 19 wherein the genetic material which functionally complements the chromosomal alteration is related to the synthesis of the bacterial cell wall.

21. The plasmid of claim 20 wherein the genetic material encodes an enzyme that catalyzes a step in the biosynthesis of said cell wall.

25

22. The plasmid of claim 21 wherein said enzyme catalyzes a reaction selected from the group consisting of the addition of L-alanine to a cell wall polysaccharide (L-Ala-adding enzyme); the addition of D-glutamic acid to the

L-alanine attached to the polysaccharide chain (*murD*); the addition of DAP to the L-alanine/D-glutamic acid dipeptide (*murE*); and the addition of D-alanine to the L-alanine/D-glutamic acid/dap tripeptide (*murF*).

5 23. A bacterial cell having its chromosome irreversibly altered so that when the bacterial cells are propagated under suitable conditions, the viability of the bacterial cells is dependent on the plasmids of any of claims 19-22, and which further includes said plasmid.

10 24. A method to replicate DNA contained in a plasmid which method comprises culturing the cells of claim 23 under conditions wherein the viability of said cells is dependent on the presence of said plasmid in said cells.

 25. A method to provide a desired DNA to a eukaryotic host organism or
15 cell which method comprises administering to said host organism or cell a DNA prepared by the method of claims 6, 14 or 24.

 26. A method to prepare prokaryotic cells containing replicated plasmid
DNA, which method comprises culturing the cells of claims 5, 13 or 23 under
20 conditions wherein the viability of said cells is dependent on the presence of said plasmid in said cells; and recovering the cells.

 27. A method to provide a desired DNA to eukaryotic host which method
comprises administering cells prepared by the method of claim 26 to said host.

25

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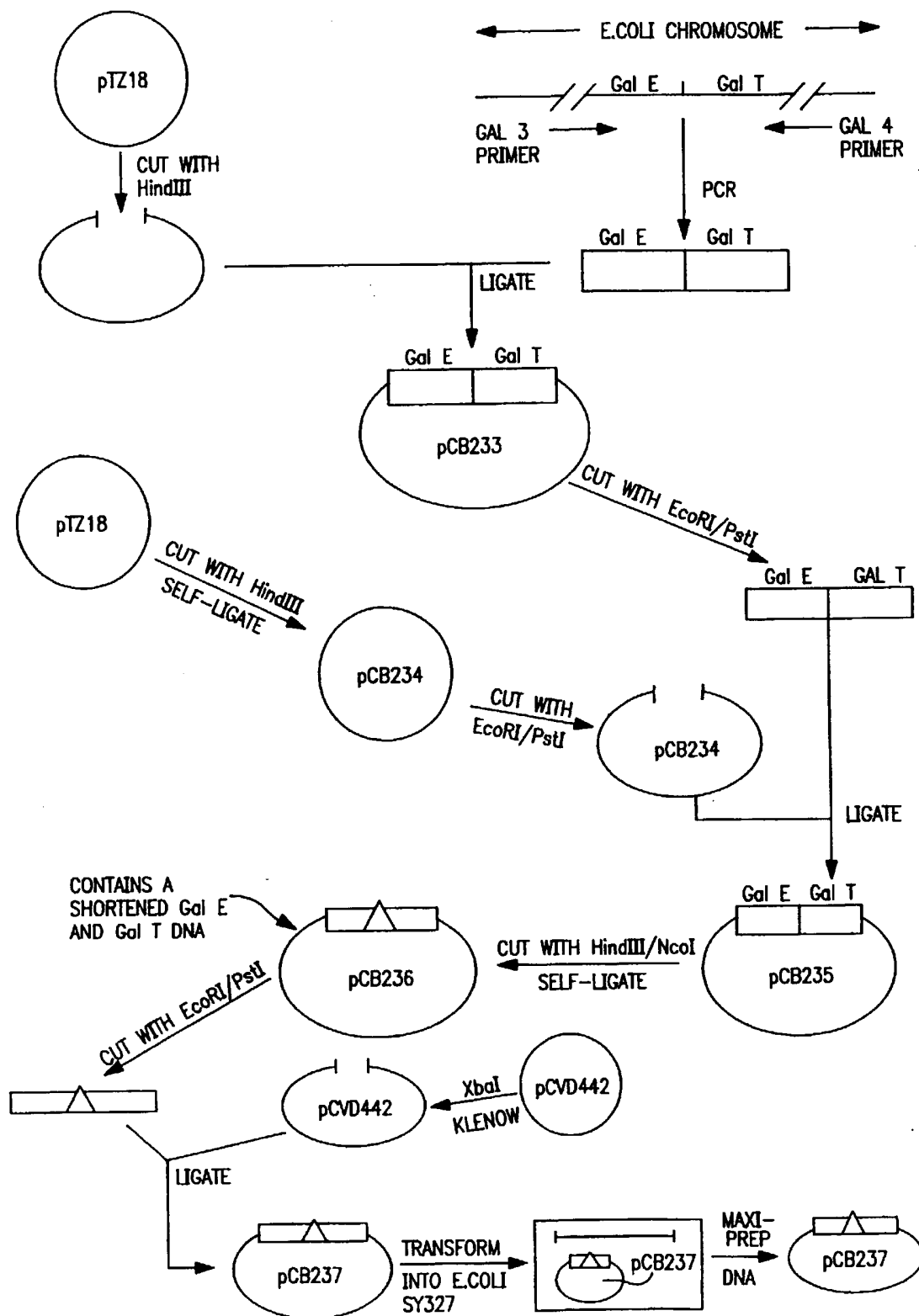


FIG. 1

SUBSTITUTE SHEET (RULE 26)

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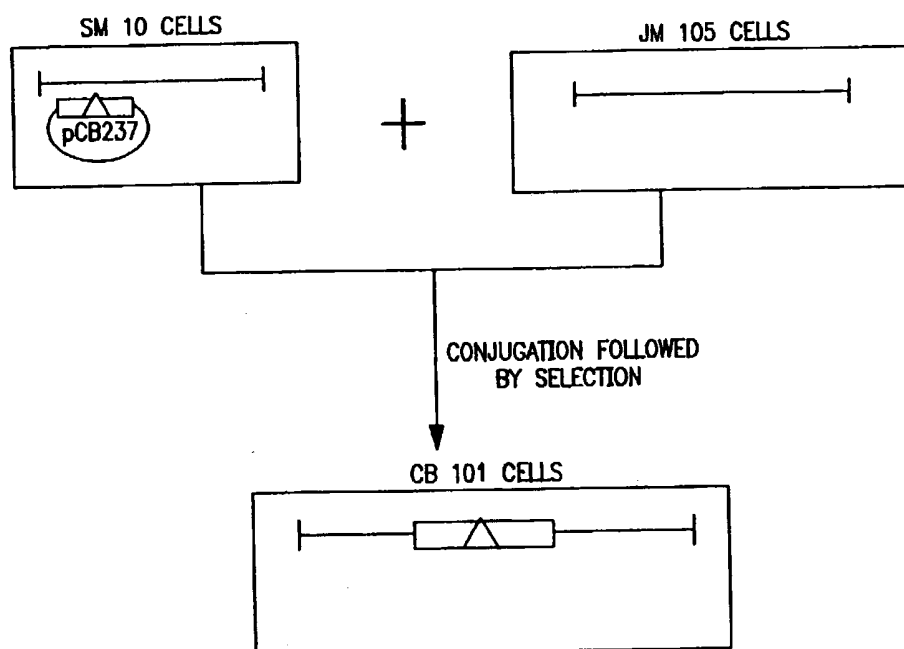
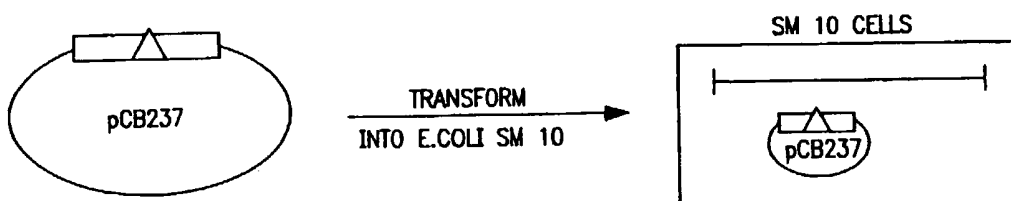


FIG. 2

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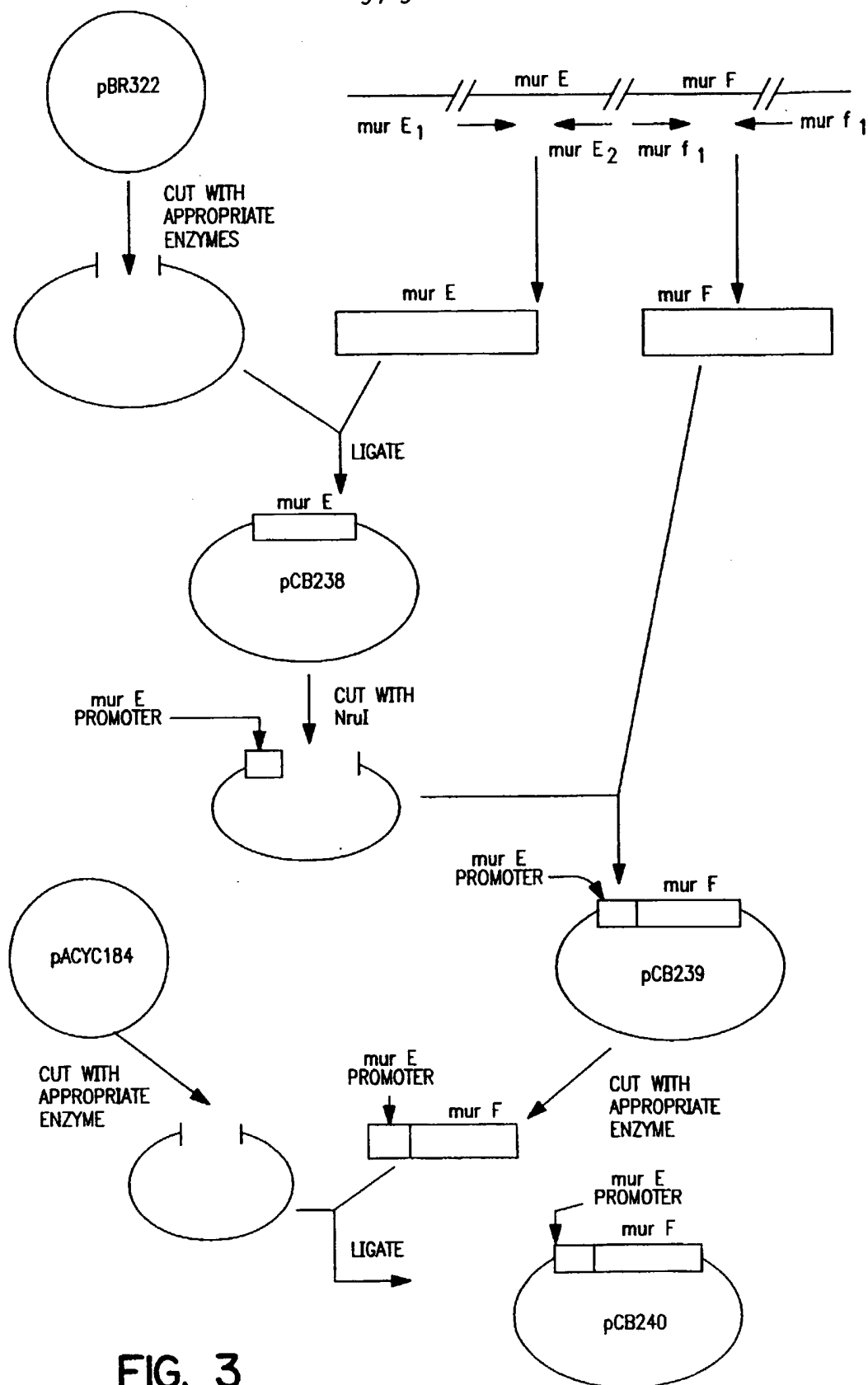


FIG. 3

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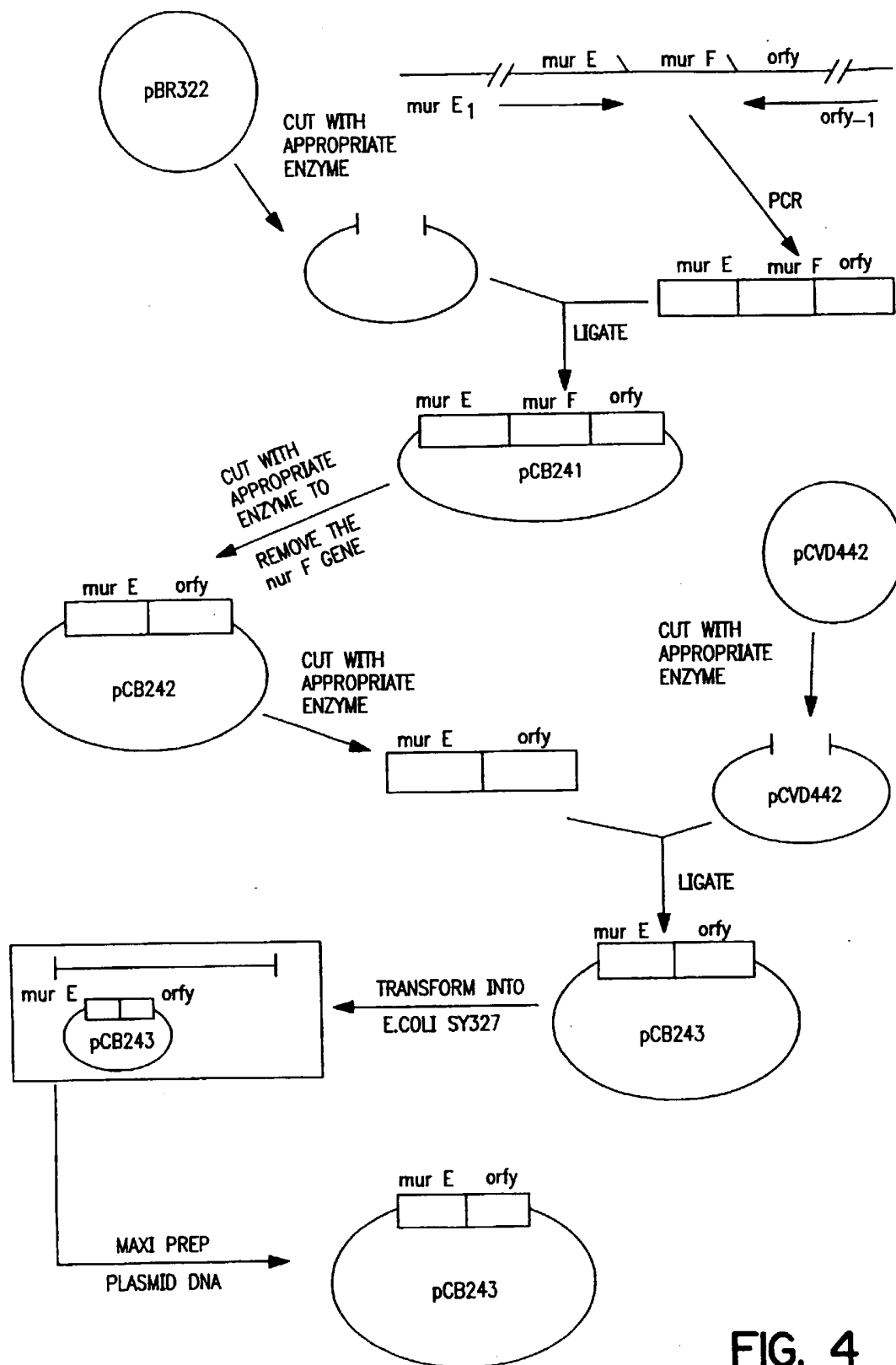


FIG. 4

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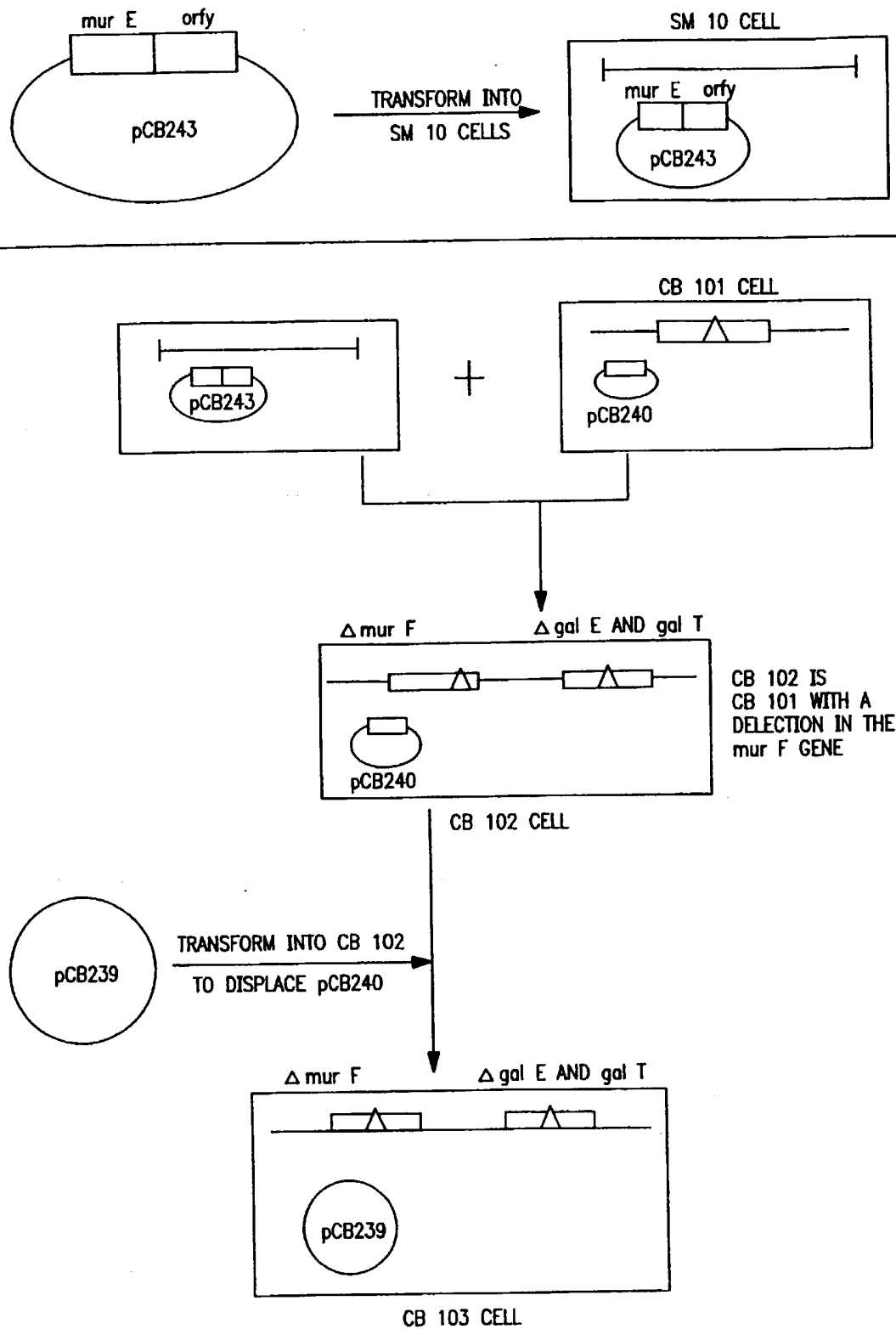
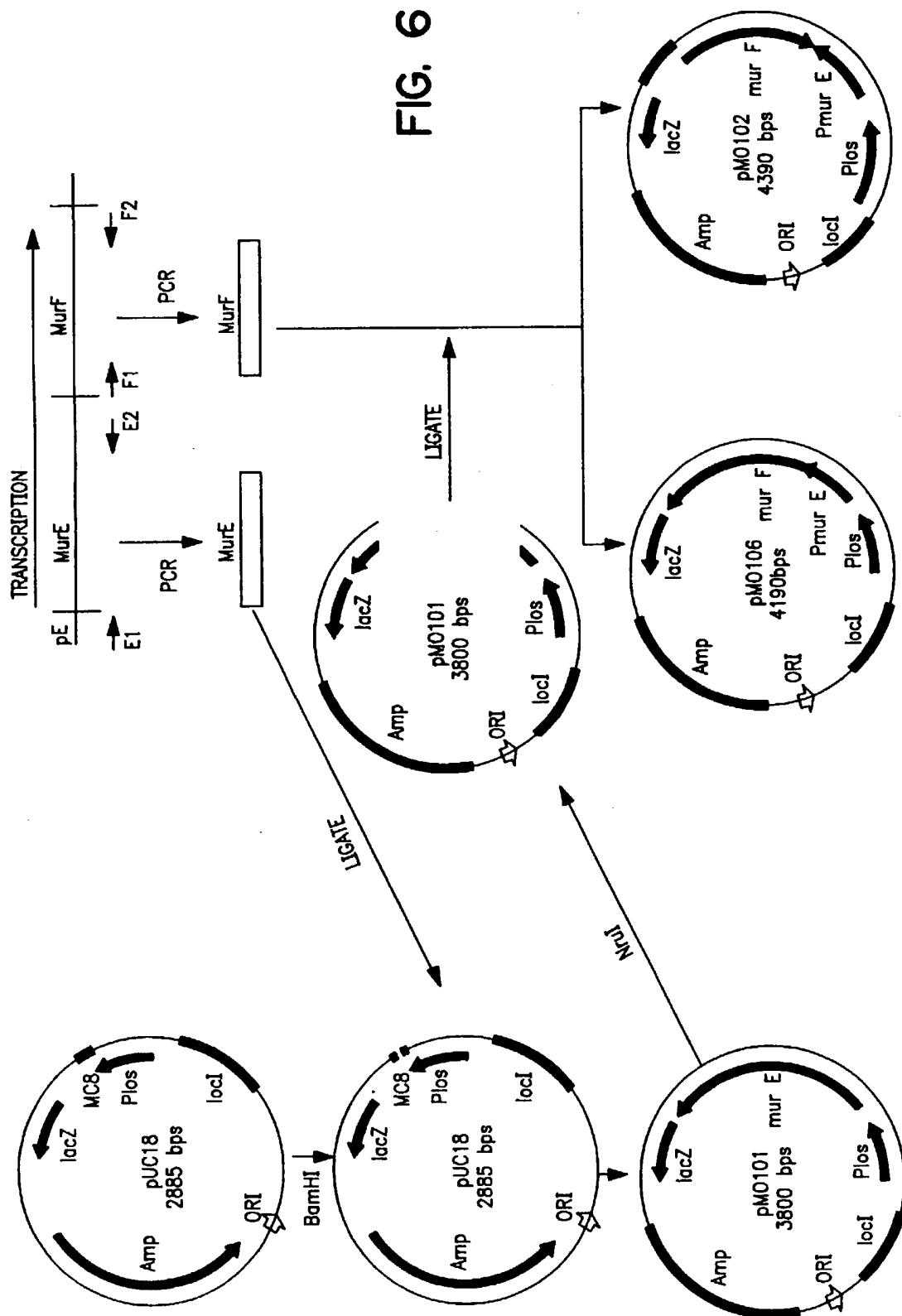


FIG. 5

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FIG. 6



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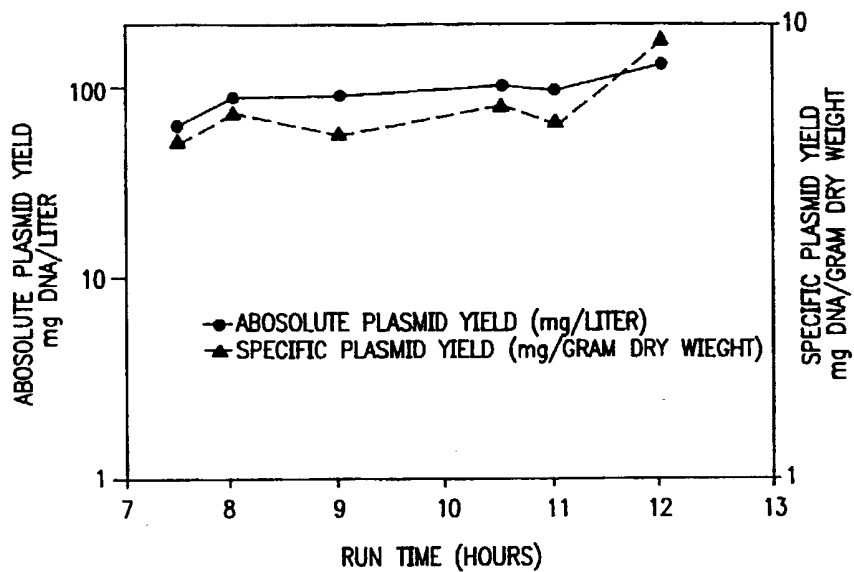


FIG. 7

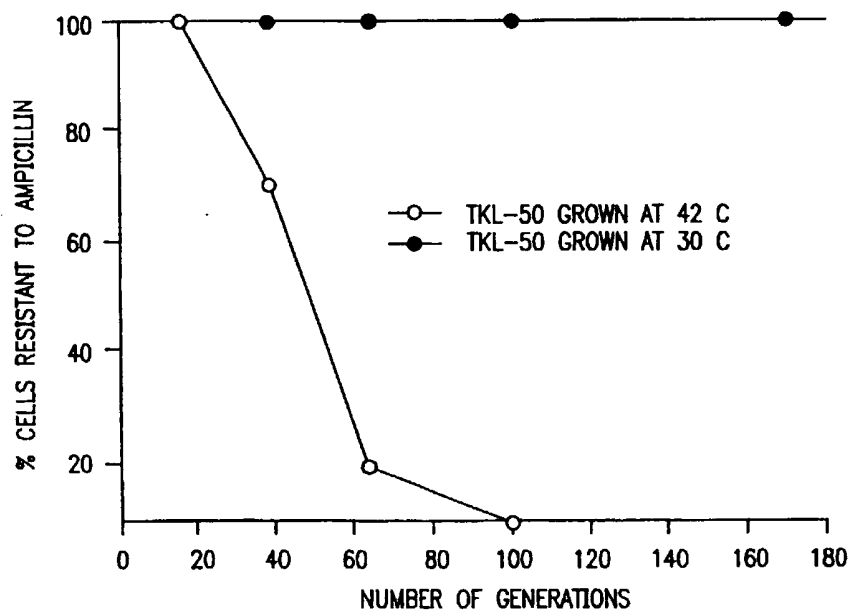


FIG. 8

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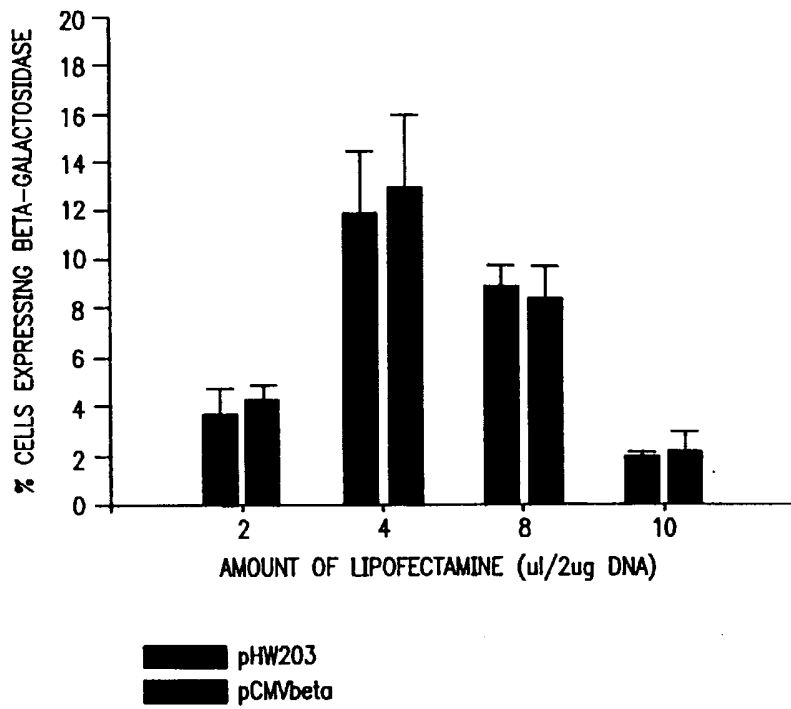


FIG. 9

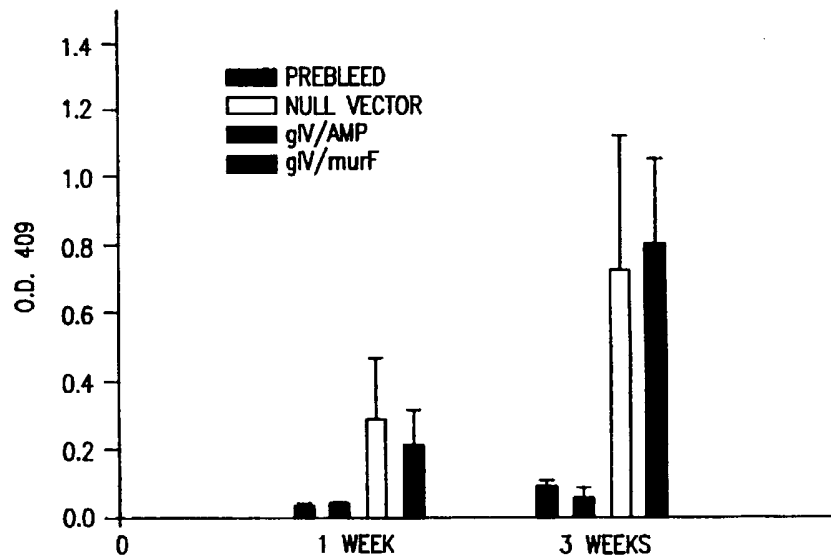


FIG. 10

